



# Suboptimal protection against H5N1 highly pathogenic avian influenza viruses from Vietnam in ducks vaccinated with commercial poultry vaccines



Ra Mi Cha<sup>a</sup>, Diane Smith<sup>a</sup>, Eric Shepherd<sup>a</sup>, C. Todd Davis<sup>b</sup>, Ruben Donis<sup>b</sup>, Tung Nguyen<sup>c,d</sup>, Hoang Dang Nguyen<sup>d</sup>, Hoa Thi Do<sup>d</sup>, Ken Inui<sup>d</sup>, David L. Suarez<sup>a</sup>, David E. Swayne<sup>a</sup>, Mary Pantin-Jackwood<sup>a,\*</sup>

<sup>a</sup> Exotic and Emerging Avian Viral Diseases Unit, Southeast Poultry Research Laboratory, USDA-Agricultural Research Service, 934 College Station Road, Athens, GA 30605, USA

<sup>b</sup> Influenza Division, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA

<sup>c</sup> Graduate School, Hanoi University of Agriculture, Hanoi, Viet Nam

<sup>d</sup> National Centre for Veterinary Diagnosis, Department of Animal Health, Hanoi, Viet Nam

## ARTICLE INFO

### Article history:

Received 11 March 2013

Received in revised form 1 August 2013

Accepted 13 August 2013

Available online 28 August 2013

### Keywords:

Ducks

H5N1 highly pathogenic avian influenza

Vaccine

Protection

## ABSTRACT

Domestic ducks are the second most abundant poultry species in many Asian countries including Vietnam, and play a critical role in the epizootiology of H5N1 highly pathogenic avian influenza (HPAI) [FAO]. In this study, we examined the protective efficacy in ducks of two commercial H5N1 vaccines widely used in Vietnam; Re-1 containing A/goose/Guangdong/1/1996 hemagglutinin (HA) clade 0 antigens, and Re-5 containing A/duck/Anhui/1/2006 HA clade 2.3.4 antigens. Ducks received two doses of either vaccine at 7 and at 14 or 21 days of age followed by challenge at 30 days of age with viruses belonging to the HA clades 1.1, 2.3.4.3, 2.3.2.1.A and 2.3.2.1.B isolated between 2008 and 2011 in Vietnam. Ducks vaccinated with the Re-1 vaccine were protected after infection with the two H5N1 HPAI viruses isolated in 2008 (HA clades 1.1 and 2.3.4.3) showing no mortality and limited virus shedding. The Re-1 and Re-5 vaccines conferred 90–100% protection against mortality after challenge with the 2010 H5N1 HPAI viruses (HA clade 2.3.2.1.A); but vaccinated ducks shed virus for more than 7 days after challenge. Similarly, the Re-1 and Re-5 vaccines only showed partial protection against the 2011 H5N1 HPAI viruses (HA clade 2.3.2.1.A and 2.3.2.1.B), with a high proportion of vaccinated ducks shedding virus for more than 10 days. Furthermore, 50% mortality was observed in ducks vaccinated with Re-1 and challenged with the 2.3.2.1.B virus. The HA proteins of the 2011 challenge viruses had the greatest number of amino acid differences from the two vaccines as compared to the viruses from 2008 and 2009, which correlates with the lesser protection observed with these viruses. These studies demonstrate the suboptimal protection conferred by the Re-1 and Re-5 commercial vaccines in ducks against H5N1 HPAI clade 2.3.2.1 viruses, and underscore the importance of monitoring vaccine efficacy in the control of H5N1 HPAI in ducks.

Published by Elsevier Ltd.

## 1. Introduction

The Asian lineage H5N1 highly pathogenic avian influenza (HPAI) viruses continue to circulate and cause great economic losses in poultry in Southeast Asia, the Middle East and Africa. The incidence of H5N1 HPAI infections is monitored globally due to their impact on avian health and production, as well as the sporadic zoonotic infections that raise pandemic concerns. Since the first virus isolation in 1996 from a goose in the Guangdong province

in China [1,2], H5N1 HPAI viruses have continued to evolve by point mutations and genetic reassortment events that affect their antigenicity and other phenotypic characteristics [3–6].

Historically, ducks naturally or experimentally infected with HPAI viruses developed only subclinical to mild disease [7–10]. A change was recognized beginning in 2002, when many emerging Asian lineage H5N1 HPAI viruses were shown to cause high mortality in ducks [11–16] however the reason for this increase in pathogenicity has not been clearly determined and appears to be multigenic in nature [17–21]. Although some H5N1 HPAI viruses can cause severe disease in ducks, a proportion of infected ducks remain clinically normal, and both sick and asymptomatic infected ducks shed virus into the environment providing opportunities for outbreaks in any exposed susceptible avian population.

\* Corresponding author. Tel.: +1 706 546 3419; fax: +1 706 546 3161.

E-mail addresses: [Mary.Pantin-Jackwood@ars.usda.gov](mailto:Mary.Pantin-Jackwood@ars.usda.gov), [mpantinj@gmail.com](mailto:mpantinj@gmail.com) (M. Pantin-Jackwood).

The duration and amount of virus shed will depend on many factors including the ducks' age and species, and the virulence of the H5N1 HPAI virus strain, with infected surviving ducks commonly shedding virus for more than seven days [22–24]. Asymptomatic infected wild migratory ducks are suspected of contributing to the spread of H5N1 HPAI viruses from Asia to other parts of the world [25–27], whereas free-range and backyard domestic ducks play a major role in perpetuating this virus in agricultural systems and in nature [23,26,28]. Free-range, as well as backyard domestic ducks have been associated with disseminating H5N1 HPAI viruses between premises [26,29–31]. Given the widespread infection of waterfowl with H5N1 HPAI viruses in certain parts of the world, reducing the risk of virus infection in domestic ducks is considered crucial for controlling the spread of H5N1 HPAI [23,32–35]. Vaccination can decrease infection rates and reduce viral shedding among infected animals, especially when enforcement of biocontainment measures is impractical. However, there is insufficient information on the efficacy of H5N1 HPAI vaccination in domestic duck species to guide disease control programs.

Vietnam is one of the countries most affected by H5N1 HPAI and the disease has remained enzootic in poultry since 2003 [36,37]. Vietnam has a highly dense domestic duck population which has been recognized as a primary factor in the spread and persistence of H5N1 HPAI in this country [37–39]. Vaccination of poultry against H5N1 HPAI, including domestic ducks, is part of the disease control strategy in Vietnam [37]. The Re-1 vaccine was used extensively to vaccinate poultry in Vietnam but was replaced in 2008 with the Re-5 vaccine, which contained antigens from a more recent field virus [40]. Despite efforts to control H5N1 HPAI in Vietnam, eradication has not been achieved, due in part to the introduction and spread of new viruses with distinct antigenic properties. The efficacy of vaccines in use should be tested against newly emerging viruses.

The objective of this study was to examine the protective efficacy of two commercial inactivated vaccines used in Vietnam to vaccinate domestic ducks against H5N1 HPAI. Three similar experiments were conducted to assess clinical and virus shedding outcomes in Re-1 or Re-5-vaccinated ducks challenged with different H5N1 HPAI viruses isolated in Vietnam in 2008, 2010 and 2011.

## 2. Materials and methods

### 2.1. Viruses and vaccines

The following H5N1 HPAI viruses were obtained from the National Center for Veterinary Diagnosis, Vietnam, and the Centers for Disease Control and Prevention, GA: A/chicken/Vietnam/NCVD-117/2008 (VN117/08) (HA clade 1.1), A/chicken/Vietnam/NCVD-185/2008 (VN185/08) (HA clade 2.3.4.3); A/chicken/Vietnam/NCVD-398/2010 (VN398/10) (HA clade 2.3.2.1.A), A/chicken/Vietnam/NCVD-421/2010 (VN421/10) (HA clade 2.3.2.1.A); A/chicken/Vietnam/NCVD-675/2011 (VN675/11) (HA clade 2.3.2.1.A) and A/duck/Vietnam/NCVD-672/2011 (VN672/11) (HA clade 2.3.2.1.B). The commercial inactivated H5N1 Re-1 and Re-5 vaccines, previously and currently used in Vietnam, produced by Harbin Veterinary Research Institute (People's Republic of China) were used to vaccinate the ducks. The Re-1 and Re-5 viruses were produced by reverse genetics to remove the polybasic amino acid cleavage site of HA. The HA and NA genes of Re-1 were from A/goose/Guangdong/1/1996 (HA clade 0), and those of Re-5 were from A/duck/Anhui/1/2006 (HA clade 2.3.4) [40]. All experiments using H5N1 HPAI viruses were performed in biosecurity level-3 enhanced facilities at the Southeast Poultry Research Laboratory (SEPRL).

### 2.2. Animal experiments

Three similar experiments were conducted (Table 1). Pekin ducks (*Anas platyrhynchos* var. *domestica*) were obtained at one day of age from a commercial hatchery. Serum samples were collected from 15 ducks in each experiment to ascertain that the birds were serologically negative for antibodies to the NP protein of influenza A viruses as determined by the commercial ELISA test FlockCheck AI (Idexx Laboratories, Westbrook, ME). One-week-old ducks (10 ducks/group) were vaccinated subcutaneously in the nape of the neck with 0.5 ml of Re-1 or Re-5 vaccine. An identical second vaccine dose was given at 14 days of age (experiment I) or at 21 days of age (experiments II and III). These vaccination schedules were based on our previous studies to evaluate responses with two doses of vaccine given before one month of age [41,42]. Additional groups of six to nine ducks in each experiment were not vaccinated and served as challenge controls. At 30 days of age, blood samples were collected, and all ducks were inoculated intranasally with 10<sup>5.0</sup> EID<sub>50</sub> of the different H5N1 HPAI challenge viruses. Ducks were observed daily for clinical signs and mortality. Oropharyngeal and cloacal swabs were collected at different days post challenge (dpc) for determining viral shedding. At the end of the experiments (9 dpc for experiment I; 15 dpc for experiment II; 10 dpc for experiment III) blood was collected from all surviving ducks for antibody assays and ducks were euthanized by injection of sodium pentobarbital (5 g/ml). All bird experiments were approved by the SEPRL Institutional Animal Care and Use Committee.

### 2.3. Serology

Hemagglutination inhibition (HI) tests were performed to determine antibody responses against vaccine and virus challenge in the serum samples collected before challenge and at 9, 15 and 10 dpc [43]. A/goose/Hong Kong/437-6/1999 (H5N1) and A/Anhui/1/2005 (H5N1) were used as HI antigens, serving as surrogate for the homologous viruses from the Re-1 and Re-5 vaccines which are not available from the manufacturer. HI titers are reported as log<sub>2</sub> values, with 3 log<sub>2</sub> being the minimum titer considered as positive.

### 2.4. Determination of virus shedding

Oropharyngeal and cloacal swabs were collected in sterile brain heart infusion medium and kept frozen at -70 °C. Viral RNA was extracted using Trizol LS reagent (Invitrogen, Calsbad, CA) and the MagMAX AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX) [44]. Quantitative real time RT-PCR (qRT-PCR) was performed as previously described [45] with modifications. Briefly, qRT-PCR targeting the influenza M gene was conducted using AgPath-ID one-step RT-PCR Kit (Ambion) and the ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Calsbad, CA). A reverse primer specifically redesigned for these Vietnam H5N1 viruses was used (5'-TCCTGCAAAGACATCTCAAGTTCTGCCG-3'). For viral quantification, a standard curve was established with viral RNA extracted from the same titrated challenge virus [41]. Results were reported as EID<sub>50</sub>/ml equivalents and the detection limit for calculated results was 10<sup>1.5</sup> EID<sub>50</sub>/ml per reaction.

### 2.5. HA gene sequencing and phylogenetic analysis

HA gene sequencing was performed as previously described [18] with modifications. The HA genes were amplified using a One-Step RT-PCR Kit (Qiagen, Valencia, CA) with specific HA primers described previously [18]. The RT-PCR products were purified using QIAquick gel extraction Kit (Qiagen) after agarose gel electrophoresis. Sequencing was performed with HA gene specific primers (available upon request) using the ABI BigDye

**Table 1**

Morbidity, mortality and mean death time of ducks vaccinated using Re-1 or Re-5 vaccines and challenged with different H5N1 HPAI viruses.

Experiment	Groups			Morbidity	Mortality	Mean death time (days)
		Vaccine	Challenge			
I	Non-vaccinated	VN/117/08 <sup>a</sup>	9/9	9/9		5.1 <sup>A</sup>
		VN/185/08 <sup>b</sup>	8/8	8/8		4.5 <sup>A</sup>
	Re-1 vaccinated	VN/117/08	0/10 <sup>e</sup>	0/10 <sup>e</sup>		— <sup>B</sup>
		VN/185/08	1/10 <sup>e</sup>	0/10 <sup>e</sup>		— <sup>B</sup>
II	Non-vaccinated	VN/398/10 <sup>c</sup>	8/8	8/8		5.4 <sup>A</sup>
		VN/421/10 <sup>c</sup>	8/8	8/8		4.0 <sup>A</sup>
	Re-1 vaccinated	VN/398/10	0/10 <sup>e</sup>	0/10 <sup>e</sup>		— <sup>B</sup>
		VN/421/10	0/10 <sup>e</sup>	0/10 <sup>e</sup>		— <sup>B</sup>
	Re-5 vaccinated	VN/398/10	3/10 <sup>e</sup>	0/10 <sup>e</sup>		— <sup>B</sup>
III	Non-vaccinated	VN/675/11 <sup>c</sup>	2/6	2/6		6.0 <sup>A</sup>
		VN/672/11 <sup>d</sup>	6/6	6/6		3.5 <sup>A</sup>
	Re-1 vaccinated	VN/675/11	1/10	0/10		— <sup>A</sup>
		VN/672/11	5/10	5/10		5.4 <sup>A</sup>
	Re-5 vaccinated	VN/675/11	0/10	0/10		— <sup>A</sup>
		VN/672/11	0/10 <sup>e</sup>	0/10 <sup>e</sup>		— <sup>B</sup>

<sup>a</sup> HA clade 1.1.

<sup>b</sup> HA clade 2.3.4.3.

<sup>c</sup> HA clade 2.3.2.1.A.

<sup>d</sup> HA clade 2.3.2.1.B; — = not applicable.

<sup>e</sup> Significant difference in number of birds presenting morbidity and mortality compared to non-vaccinated group; Fisher's exact test,  $P<0.05$ . Different superscript uppercase letters denote significant difference for survival when compared to the non-vaccinated group (Log rank) Mantel-Cox test,  $P<0.05$ .

Terminator 1.1 reaction mix and the ABI 3730XL automated DNA sequencer (Applied Biosystems). The sequence of the vaccine seed viruses was obtained from GenBank and/or GISAID databases. The gene and protein sequence from each virus was compared with the vaccine virus sequence using DNASTAR program (Madison, WI) and BioEdit. Phylogenetic trees were constructed by neighbor-joining analysis of HA gene nucleotide alignments using MEGA5. The GISAID accession numbers for the HA sequences of the challenge viruses are: A/chicken/Vietnam/NCVD-185/2008: EPI284451; A/chicken/Vietnam/NCVD-117/2008: EPI284449; A/chicken/Vietnam/NCVD-398/2010: EPI284468; A/chicken/Vietnam/NCVD-675/2011: EPI405128 A/chicken/Vietnam/NCVD-421/2010: EPI284470; and A/duck/Vietnam/NCVD-672/2011: EPI330995.

## 2.6. Statistical analysis

Data was analyzed using Prism v.5.01 software (GraphPad Software Inc., La Jolla, CA, USA). The survival rate data was analyzed using the Mantel-Cox Log-Rank test. Morbidity, mortality, and number of birds shedding virus were tested for statistical significance using Fisher's exact test. Significant difference for mean viral titers between vaccinated groups and non-vaccinated controls was done using the unpaired *t* test. A  $P$ -value of  $<0.05$  was considered to be significant.

## 3. Results

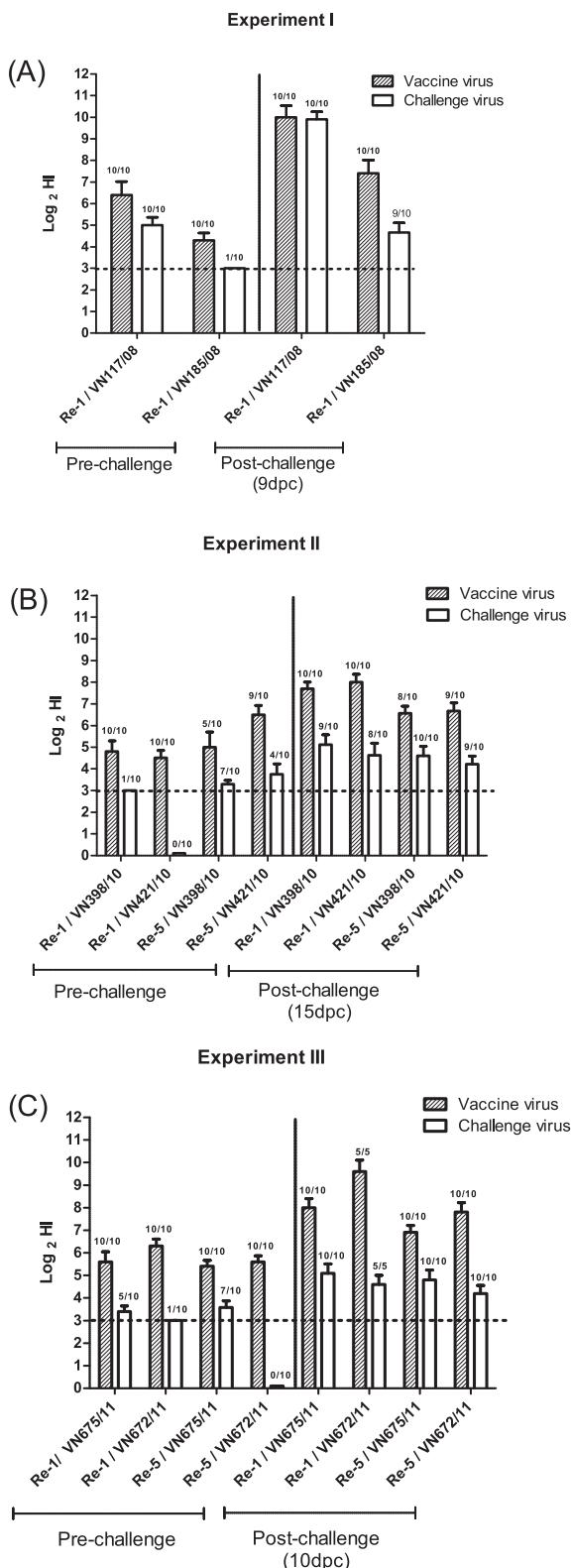
### 3.1. Protective efficacy of H5N1 vaccines: morbidity and mortality outcomes

Five of the six H5N1 HPAI viruses used to challenge vaccine immunity in ducks were lethal for non-vaccinated control ducks, except with the VN 675/11 virus (HA clade 2.3.2.1.A) in experiment III. This virus caused 33% mortality with a mean death time (MDT) of 6 days (Table 1). The most prominent clinical signs in non-vaccinated H5N1-challenged ducks were lethargy, anorexia, prostration, and neurological signs, similar to previous reports [16,17]. The survival of the vaccinated and non-vaccinated ducks is

shown in Supplementary Fig. 1. The mortality of ducks vaccinated with Re-1 and Re-5 and challenged with viruses from 2008 and 2010 was significantly reduced when compared to non-vaccinated ducks ( $P<0.05$ ). These ducks were significantly protected against H5N1 HPAI with minimal mortality and clinical signs (transient mild lethargy and anorexia). In contrast, the Re-1-vaccinated ducks were only partially protected from A/VN/672/11 (HA clade 2.3.2.1.B) virus challenge; these animals developed clinical signs similar to those of non-vaccinated controls, and 50% of the animals died with a MDT of 5.4 days. However, Re-5-vaccinated ducks challenged with VN/672/11 were fully protected against mortality and clinical signs. Re-1 or Re-5-vaccinated ducks challenged with VN/675/11 (HA clade 2.3.2.1.A) did not show clinical signs or mortality, but this was not significantly different than what seen with the non-vaccinated controls.

### 3.2. Serum antibody responses to vaccination and infection

Serological responses to vaccination and virus challenge were assessed by HI assays which detect antibodies specific to the HA glycoprotein (Fig. 1). The majority of vaccinated ducks developed pre-challenge HI titers ( $\geq 4 \log_2$ ) when using the vaccine virus antigen in the test (surrogate homologous virus), indicating most vaccinated ducks seroconverted. However, some vaccinated ducks had HI titers under the limit considered positive ( $\leq 3 \log_2$ ). The antibody response to the challenge viruses (heterologous virus relative to the vaccine) measured on samples collected before challenge, revealed differential HI responses; 100% of Re-1-vaccinated ducks showed cross HI responses to the VN 117/08 (HA clade 1) virus antigen with 5 mean  $\log_2$  titer, in contrast, only one duck from the group challenge with the VN 185/08 (HA clade 2.3.4) was positive. Lower levels of HI cross reactivity was observed with samples from Re-1-vaccinated ducks where 0–50% were positive when using the 2010 and 2011 viruses in the test (HA clade 2.3.2.1) and ducks vaccinated with Re-5 were 0–70% positive. As expected, high post-challenge titers were observed in more than 80% of the vaccinated challenged ducks that survived in all three experiments when using the vaccine virus in the HI tests ( $\geq 7 \log_2$ ). 80–100% of the vaccinated challenged ducks that survived had positive titers when using the challenge



**Fig. 1.** Mean HI titer ( $\log_2$ ) in Re-1 or Re-5 vaccinated-challenged ducks. Duck were vaccinated with the Re-1 or Re-5 vaccines two times, at the one week of age and 7 or 14 days later. Serum was collected at 30 days of age, before challenge, and 9–15 days post-challenge to examine the antibody responses. The number of ducks with positive HI titer is shown above each group [number of positive sera ( $\geq 3 \log_2$ )/total number of sera tested]. Bars represent standard deviation within groups.

virus in the HI tests ( $\geq 4\log_2$ ) indicating exposure and replication of the challenge viruses.

### 3.3. Protective efficacy of H5N1 vaccines: virus shedding outcomes

Virus shedding was monitored by quantification of viral RNA in oropharyngeal and cloacal samples collected at different time points after virus challenge. Limited virus shedding was noted at 3 and 5 dpc in ducks vaccinated with Re-1 and challenged with either of the two H5N1 HPAI viruses from 2008 (Table 2). Significantly less number of vaccinated ducks were shedding virus, and in less quantity, when compared to the non-vaccinated controls at 3 dpc. No virus shedding was detected at 7 dpc from any of the vaccinated ducks. In contrast, 40–60% of ducks vaccinated with Re-1 and challenged with either one of the 2010 viruses were still shedding at 7 dpc (Table 3). The Re-5 conferred more effective protection against 2010 viruses, with only 10–20% of ducks shedding at this time point. Of note, 40–90% of ducks vaccinated with Re-1 or Re-5 and challenged with the VN 675/11 virus were still shedding virus at 10 dpc (Table 4). 50–100% of ducks vaccinated with either vaccine and challenged with the VN/672/11 virus were still shedding virus at 10 dpc. Nonetheless, the vaccines significantly reduced the amount oropharyngeal virus shedding when compared to controls.

### 3.4. Amino acid changes in the HA sequences of challenge viruses

The HA gene sequences of the two vaccines were compared with those from the H5N1 HPAI challenge viruses to determine the similarity among them (Table 5; Supplemental Table 6). The challenge viruses were isolated in different years and belong to different HA clades or subclades (Supplemental Fig. 2). The viruses isolated in 2008, HA clades 1.1 and 2.3.4.3, showed 94.6% and 94.5% amino acid (aa) similarity with the Re-1 vaccine and had 22 aa changes in the mature HA1 proteins but only 21 or 3 substitutions, respectively, compared to Re-5. The viruses from 2010, both HA clade 2.3.2.1.A, showed 92.1% and 92.3% aa similarity with the Re-1 vaccine (31 and 32 aa in HA1) and 94.5% and 94.4% with the Re-5 vaccine (21 and 22 aa in HA1). The viruses from 2011, HA clade 2.3.2.1.A and 2.3.2.1.B, showed the least similarity with the vaccines, 92.4% and 91.6% with Re-1 (30 and 40 HA1 substitutions, respectively), and 94.9% and 93.1% with Re-5 (21 and 35 HA1 substitutions, respectively). The aa differences between the vaccines and challenge viruses HA were mostly found in the HA1 subunit protein region with a high proportion of these occurring in previously described antigenic sites. In addition to these substitutions, several changes between vaccine strains and challenge viruses were predicted to result in gain or loss of N-linked glycosylation at position 154–156. Relative to Re-1, clade 2.3.4 and 1.1 viruses possess a putative gain in glycosylation. Relative to Re-5, the clade 2.3.2.1 viruses demonstrate a loss of glycosylation. The lowest aa similarity (91.6%) and largest number of HA1 substitutions was found between the Re-1 vaccine and the VN 672/11 virus, this combination also showed the least protection in vaccinated ducks.

## 4. Discussion

In this study we examined the protective efficacy of two commercial inactivated vaccines in domestic ducks against infection with increasingly divergent H5N1 HPAI viruses isolated in Vietnam in 2008, 2010 and 2011. Despite control efforts, H5N1 HPAI viruses continue to circulate in Vietnam within various poultry populations, including domestic ducks. Introduction of new H5N1 genotypes as well as local evolution of viruses through mutation and reassortment continue to occur [6,46]. In the present study, we used viruses belonging to different HA clades and all, with the

**Table 2**

Experiment I, virus shedding. Ducks were vaccinated with the Re-1 vaccine and challenged with VN117/08 or VN/185/08 H5N1 HPAI viruses.

Groups		Virus titers from swabs collected at different days post-challenge (dpc) # of positive ducks/total ducks <sup>a</sup> (Mean titer ± SD) <sup>b</sup>					
Vaccination	Challenge virus	3 dpc OP <sup>c</sup>	C <sup>d</sup>	5 dpc OP	C	7 dpc OP	C
Non-vaccinated	VN 117/08	9/9 (4.7 ± 0.3) <sup>A</sup>	6/9 (2.0 ± 0.5) <sup>A</sup>	—	—	—	—
Re-1	VN 117/08	3/8 <sup>e</sup> (2.4 ± 1.2) <sup>B</sup>	0/8 <sup>e</sup> (—) <sup>B</sup>	4/10 (2.2 ± 1.4)	3/10 (1.5 ± 0.8)	0/10 (—)	0/10 (—)
Non-vaccinated	VN 185/08	8/8 (4.9 ± 1.0) <sup>A</sup>	7/8 (2.7 ± 0.8) <sup>A</sup>	—	—	—	—
Re-1	VN 185/08	2/10 <sup>e</sup> (2.5 ± 1.7) <sup>B</sup>	1/10 <sup>e</sup> (2.7) <sup>B</sup>	1/10 (1.6 ± 0.6)	0/10 (1.0 ± 0.1)	0/10 (—)	0/10 (—)

<sup>a</sup> Swab samples were taken from all ducks remaining at each day post-challenge.<sup>b</sup> Log<sub>10</sub> EID<sub>50</sub>-equivalents were determined by qRT-PCR, numbers in parenthesis represent the mean of the viral titers ± standard deviation.<sup>c</sup> Oropharyngeal swabs.<sup>d</sup> Cloacal swabs; — = not applicable.<sup>e</sup> Significant difference for number of positive ducks by qRT-PCR compared to non-vaccinated group, P < 0.05. Different superscript uppercase letters denote significant difference for mean viral titers between vaccinated and non-vaccinated groups, P < 0.05. For statistical purposes all swabs without viral RNA detection were given a numeric value of 1.5 Log<sub>10</sub> EID<sub>50</sub>-equivalents.

exception of one of the 2011 viruses, caused severe clinical signs and mortality in non-vaccinated control ducks. The high virulence of H5N1 HPAI viruses in young domestic ducks has been previously reported and is not exclusive to a specific HA clade [16,47].

Many studies have shown that inactivated whole virion oil-adjuvanted vaccines are effective in protecting domestic ducks against H5N1 HPAI [41,42,48–52]. The efficacy of vaccines is a key factor in the success of control programs which rely on them to reduce virus spread. From 2005–2009, 90% of the national domestic duck and 50% of national chicken populations in Vietnam were vaccinated against H5N1 and the majority of outbreaks reported in 2005–2009 were in non-vaccinated ducks [37]. However, more recently, reports have emerged in H5N1 enzootic countries of vaccinated poultry flocks with outbreaks of HPAI [53]. Our studies demonstrate that the efficacy of two inactivated vaccines routinely used in Vietnam, Re-1 and Re-5 to protect ducks against H5N1 HPAI varied depending on the virus used for challenge. Ducks vaccinated with Re-1 were well protected when challenged with viruses from 2008. A decrease in protection, however, was observed in ducks vaccinated with Re-1 or Re-5 and challenged with the viruses from 2010 and 2011, with a substantial proportion of ducks having prolonged virus shedding, and in the case of A/VN/672, some mortality. Most ducks showed detectable HI titers after the prime-boost vaccine regimen, indicating that vaccination stimulated humoral antibody responses to the surrogate homologous virus. HI titers to the challenge virus antigens were also observed, albeit lower than the homologous viruses; however, these titers were not always associated with protection conferred by vaccination. Lack of antibodies detected by HI in vaccinated birds is not a predictor of

the complete lack of immunity, as previously reported [41,48,54]. Assays that better correlate the immune response to vaccination with the outcomes of infection with wild type viruses are urgently needed [55].

The HA of the 2008 H5N1 HPAI viruses had the highest aa similarity with the Re-1 vaccine HA sequence, consistent with previous reports indicating that HA similarity between vaccine and challenge virus is often associated with higher vaccine efficacy. Interestingly, VN 117/08 and VN 185/08 belong to clade 1.1 and 2.3.4.3, respectively, but the ducks were equally well protected after vaccination with the Re-1 vaccine (HA clade 0). These results recapitulate our previous findings showing that the Re-1 vaccine protected ducks against morbidity when challenged with H5N1 HPAI viruses isolated in Vietnam 2005 and 2007 with heterologous HA clades [41,42,48]. These studies underlined the importance of antigenic similarity between the vaccine and the virus challenge on the protective efficacy in ducks, but also highlighted the importance of other factors such as the vaccination regimen, the duck species and the virulence of the challenge virus used.

Despite protection against morbidity and mortality, a large proportion of vaccinated ducks challenged with the 2010 and 2011 H5N1 HPAI viruses were shedding virus and for longer periods, suggesting inadequate protection. Although the Re-5 is a more recently updated vaccine, it did not provide superior protection to one of the clade 2.3.2.1 (group B) viruses (VN/672/11). While the Re-5 vaccine had a greater similarity to the clade 2.3.4 viruses circulating in Vietnam until 2010, these viruses were suddenly replaced by the new clade 2.3.2.1 viruses. Consequently, the replacement of Re-1 with Re-5 vaccine (clade 2.3.4) in Vietnam did not meet the objective

**Table 3**

Experiment II, virus shedding. Ducks were vaccinated with Re-1 or Re-5 vaccine and challenged with VN398/10 or VN421/10 H5N1 HPAI viruses.

Groups		Virus titers from swabs collected at different days post-challenge (dpc) # of positive ducks/total ducks <sup>a</sup> (Mean titer ± SD) <sup>b</sup>					
Vaccination	Challenge virus	3 dpc OP <sup>c</sup>	C <sup>d</sup>	5 dpc OP	C	7 dpc OP	C
Non-vaccinated	VN 398/10	8/8 (5.9 ± 0.4) <sup>A</sup>	8/8 (3.7 ± 0.5) <sup>A</sup>	5/5 (5.5 ± 0.5) <sup>A</sup>	5/5 (3.8 ± 0.4) <sup>A</sup>	3/3 (4.4 ± 0.8) <sup>A</sup>	3/3 (3.3 ± 0.5) <sup>A</sup>
Re-1	"	8/10 (3.8 ± 1.0) <sup>B</sup>	9/10 (3.5 ± 1.6) <sup>A</sup>	8/10 (3.0 ± 0.6) <sup>B</sup>	8/10 (3.0 ± 0.8) <sup>A</sup>	6/10 (2.5 ± 0.3) <sup>B</sup>	4/10 <sup>e</sup> (2.2 ± 0.5) <sup>B</sup>
Re-5	"	8/10 (3.2 ± 1.1) <sup>B</sup>	5/10 <sup>e</sup> (2.5 ± 0.2) <sup>B</sup>	4/10 <sup>e</sup> (2.3 ± 0.5) <sup>B</sup>	2/10 <sup>e</sup> (2.3 ± 0.3) <sup>B</sup>	2/10 <sup>e</sup> (1.8 ± 0.7) <sup>B</sup>	2/10 <sup>e</sup> (2.2 ± 0.1) <sup>B</sup>
Non-vaccinated	VN 421/10	5/5 (6.2 ± 0.5) <sup>A</sup>	5/5 (4.0 ± 0.7) <sup>A</sup>	3/3 (5.6 ± 0.3) <sup>A</sup>	3/3 (4.5 ± 1.3) <sup>A</sup>	1/1 (4.6) <sup>A</sup>	1/1 (3.9) <sup>A</sup>
Re-1	"	10/10 (3.7 ± 1.2) <sup>B</sup>	10/10 (3.1 ± 0.4) <sup>A</sup>	3/10 (2.4 ± 0.1) <sup>B</sup>	4/10 <sup>e</sup> (2.5 ± 0.3) <sup>B</sup>	2/10 (2.1 ± 0.4) <sup>B</sup>	4/10 <sup>e</sup> (2.3 ± 0.2) <sup>B</sup>
Re-5	"	9/10 (3.2 ± 1.8) <sup>B</sup>	6/10 (2.2 ± 0.4) <sup>B</sup>	3/10 (2.4 ± 0.1) <sup>B</sup>	5/10 (2.4 ± 0.3) <sup>B</sup>	1/10 (1.9) <sup>B</sup>	1/10 (2.0) <sup>B</sup>

<sup>a</sup> Swab samples were taken from all ducks remaining at each day post-challenge.<sup>b</sup> Log<sub>10</sub> EID<sub>50</sub>-equivalents were determined by qRT-PCR, numbers in parenthesis represent the mean of the viral titers ± standard deviation.<sup>c</sup> Oropharyngeal swabs.<sup>d</sup> Cloacal swabs.<sup>e</sup> Significant difference for number of positive ducks by qRT-PCR compared to non-vaccinated group, P < 0.05. Different superscript uppercase letters denote significant difference for mean viral titers between vaccinated and non-vaccinated groups, P < 0.05. For statistical purposes all swabs without viral RNA detection were given a numeric value of 1.5 Log<sub>10</sub> EID<sub>50</sub>-equivalents.

**Table 4** Experiment III, virus shedding. Ducks were vaccinated with Re-1 or Re-5 vaccine and challenged with VN675/11 or VN672/11 H5N1 HPAI viruses.

Groups Vaccination	Challenge virus	Virus titers from swabs collected at different days post-challenge (dpc) # of positive ducks/total ducks <sup>a</sup> (Mean titer ± SD) <sup>b</sup>					
		3 dpc			6 dpc		
		OP <sup>c</sup>	C <sup>d</sup>	OP	C	OP	C
Non-vaccinated	VN 675/11	6/6 (6.5 ± 0.7) <sup>A</sup>	6/6 (4.0 ± 0.4) <sup>A</sup>	4/4 (4.2 ± 0.3) <sup>A</sup>	4/4 (4.7 ± 1.0) <sup>A</sup>	3/4 (3.2 ± 1.6) <sup>A</sup>	4/4 (4.7 ± 0.3) <sup>A</sup>
Re-1	"	9/10 (4.4 ± 1.0) <sup>B</sup>	4/10 <sup>e</sup> (2.6 ± 0.5) <sup>B</sup>	4/10 <sup>e</sup> (2.5 ± 0.2) <sup>B</sup>	6/10 (2.4 ± 0.1) <sup>B</sup>	4/10 (2.3 ± 0.2) <sup>A</sup>	1/10 <sup>e</sup> (3.2) <sup>B</sup>
Re-5	"	7/10 (3.2 ± 0.8) <sup>B</sup>	5/10 <sup>e</sup> (2.4 ± 0.3) <sup>B</sup>	10/10 (3.4 ± 0.3) <sup>B</sup>	10/10 (3.0 ± 0.3) <sup>B</sup>	9/10 (2.9 ± 0.3) <sup>B</sup>	9/10 (3.1 ± 0.2) <sup>B</sup>
Non-vaccinated	VN 672/11	5/5 (6.9 ± 0.3) <sup>A</sup>	5/5 (4.5 ± 0.4) <sup>A</sup>	—	—	—	—
Re-1	"	10/10 (5.7 ± 0.6) <sup>A</sup>	10/10 (3.5 ± 0.5) <sup>B</sup>	6/6 (3.3 ± 0.9)	5/6 (2.3 ± 0.4)	5/5 (3.1 ± 0.5)	5/5 (3.1 ± 0.4)
Re-5	"	10/10 (4.8 ± 1.2) <sup>B</sup>	10/10 (3.0 ± 0.4) <sup>B</sup>	5/10 (2.7 ± 1.2)	2/10 (1.8 ± 0.5)	4/10 (2.1 ± 0.3)	5/10 (3.0 ± 1.1)

<sup>a</sup> Swab samples were taken from all ducks remaining at each day post-challenge.<sup>b</sup> Log<sub>10</sub> EID<sub>50</sub>-equivalents were determined by qRT-PCR, numbers in parenthesis represent the mean of the viral titers ± standard deviation.<sup>c</sup> Oropharyngeal swabs.<sup>d</sup> Cloacal swabs; — = Not applicable<sup>e</sup> Significant difference for number of positive ducks by qRT-PCR compared to non-vaccinated group, P < 0.05. Different superscript uppercase letters denote significant difference for mean viral titers between vaccinated and non-vaccinated groups, P < 0.05. For statistical purposes all swabs without viral RNA detection were given a numeric value of 1.5 Log<sub>10</sub> EID<sub>50</sub>-equivalents.**Table 5** HA sequence similarity between commercial vaccines and challenge viruses.

Experiment	Viruses	Amino acid similarity (%)	
		Re-1 A/goose/ Guangdong/1/1996 (HA clade 0)	Re-5 A/duck/ Anhui/1/2006 (HA clade 2.3.4)
I	A/Chicken/Vietnam/ NCVD-117/2008 (HA clade 1.1)	94.6	95.2
	A/Chicken/Vietnam/ NCVD-185/2008 (HA clade 2.3.4.3)	94.5	98.9
II	A/Chicken/Vietnam/ NCVD-398/2010 (HA clade 2.3.2.A)	92.1	94.5
	A/Chicken/Vietnam/ NCVD-421/2010 (HA clade 2.3.2.A)	92.3	94.4
III	A/Chicken/Vietnam/ NCVD-675/2011 (HA clade 2.3.2.A)	92.4	94.5
	A/duck/Vietnam/ NCVD-672/2011 (HA clade 2.3.2.B)	91.6	93.1

of matching the vaccine antigenic characteristics to the predominant viruses circulating in 2011 (clade 2.3.2.1). In late 2012 a new H5N1 vaccine seed strain was licensed, Re-6, which is based on a 2.3.2.1 clade virus and closer genetically to the 2.3.2.1 H5N1 viruses circulating in Vietnam in 2011.

The effectiveness of vaccines in preventing infection, disease, and transmission of antigenically divergent viruses has not been extensively studied in domestic ducks. Previous reports in chickens with H5N2 avian influenza have shown that HA sequence similarity between the vaccine and challenge virus directly relates with the ability of the vaccine to reduce virus shedding [56–58]. Other studies showed that when the vaccine and the challenge virus belong to same H5N1 subtype, and expected to have high HA homology, vaccinated ducks were completely protected against challenge virus [49,51,54]. However, other studies revealed that genetically more distant vaccines can also protect ducks against infection with H5N1 viruses [35,48,52]. Although there is a correlation between genetic and antigenic distance, it is also possible that specific genetic differences of as little as a single aa can lead to a substantial difference in antigenic distance [59]. Other variables including host differences, vaccination schedules, and the use of different adjuvants, can also affect the protection conferred by vaccines.

## 5. Conclusion

In this study, we showed clear differences in vaccine efficacy in protecting domestic ducks against H5N1 HPAI viruses circulating in Vietnam. A suboptimal protection was observed in the vaccinated ducks challenged with the 2010 and 2011 viruses. HA sequence similarity between the vaccines and the challenge viruses correlated with the vaccine efficacy in this study. To ensure adequate protection of domestic ducks against constantly changing H5N1 HPAI field strains, routine vaccine efficacy testing is required.

## Acknowledgements

The authors would like to thank Drs. Mariana Sa E. Silva and Kateri Bertran for technical assistance. This study was supported by a Specific Cooperative Agreement with the Foreign Agriculture Service of the USDA Project # #60-6612-0-024, and the Agriculture Research Service CRIS Projects 6612-32000-048 and

6612-32000-063. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2013.08.046>.

## References

- [1] Webster RG, Govorkova EA. H5N1 influenza – continuing evolution and spread. *N Engl J Med* 2006;355:2174–7.
- [2] Xu X, Subbarao K, Cox NJ, Guo Y. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology* 1999;261:15–9.
- [3] Arafa A, Suarez DL, Hassan MK, Aly MM. Phylogenetic analysis of hemagglutinin and neuraminidase genes of highly pathogenic avian influenza H5N1 Egyptian strains isolated from 2006 to 2008 indicates heterogeneity with multiple distinct sublineages. *Avian Dis* 2010;54:345–9.
- [4] Balish AL, Davis CT, Saad MD, El-Sayed N, Esmat H, Tjaden JA. Antigenic and genetic diversity of highly pathogenic avian influenza A(H5N1) viruses isolated in Egypt. *Avian Dis* 2010;54:329–34.
- [5] WHO. Continuing progress towards a unified nomenclature for the highly pathogenic H5N1 avian influenza viruses: divergence of clade 2.2 viruses. *Influenza Other Respi Viruses* 2009;3:59–62.
- [6] Nguyen T, Rivailler P, Davis CT, Thi Hoa D, Balish A, Hoang Dang N, et al. Evolution of highly pathogenic avian influenza (H5N1) virus populations in Vietnam between 2007 and 2010. *Virology* 2012;432:405–16.
- [7] Alexander DJ, Parsons G, Manvell RJ. Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks and quail. *Avian Pathol* 1986;15:647–62.
- [8] Cooley AJHVC, Philpott MS, Easterday BC, Hinshaw VS. Pathological lesions in the lungs of ducks with influenza A viruses. *Vet Pathol* 1989;26:1–5.
- [9] Perkins LE, Swayne DE. Pathogenicity of a Hong Kong-origin H5N1 highly pathogenic avian influenza virus for emus, geese, ducks, and pigeons. *Avian Dis* 2002;46:53–63.
- [10] Shortridge KF, Zhou NN, Guan Y, Gao P, Ito T, Kawaoka Y, et al. Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. *Virology* 1998;252:331–42.
- [11] Pfeiffer J, Pantin-Jackwood M, To TL, Nguyen T, Suarez DL. Phylogenetic and biological characterization of highly pathogenic H5N1 avian influenza viruses (Vietnam 2005) in chickens and ducks. *Virus Res* 2009;142:108–20.
- [12] Tang Y, Wu P, Peng D, Wang X, Wan H, Zhang P, et al. Characterization of duck H5N1 influenza viruses with differing pathogenicity in mallard (*Anas platyrhynchos*) ducks. *Avian Pathol* 2009;38:457–67.
- [13] Bingham J, Green DJ, Lowther S, Klippen J, Burggraaf S, Anderson DE, et al. Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (*Anas platyrhynchos*), with particular reference to clinical disease, tissue tropism and viral shedding. *Avian Pathol* 2009;38:267–78.
- [14] Guionie O, Guillou-Cloarec C, Courtois D, Bougeard BS, Amelot M, Jestin V. Experimental infection of Muscovy ducks with highly pathogenic avian influenza virus (H5N1) belonging to clade 2. *Avian Dis* 2010;54:538–47.
- [15] Phuong do Q, Dung NT, Jorgensen PH, Handberg KJ, Vinh NT, Christensen JP. Susceptibility of Muscovy (*Cairina Moschata*) and mallard ducks (*Anas Platyrhynchos*) to experimental infections by different genotypes of H5N1 avian influenza viruses. *Vet Microbiol* 2011;148:168–74.
- [16] Pantin-Jackwood MJ, Swayne DE. Pathogenesis and pathobiology of avian influenza virus infection in birds. *Rev Sci Tech* 2009;28:113–36.
- [17] Pantin-Jackwood MJ, Swayne DE. Pathobiology of Asian highly pathogenic avian influenza H5N1 virus infections in ducks. *Avian Dis* 2007;51:250–9.
- [18] Wasilenko JL, Arafa AM, Selim AA, Hassan MK, Aly MM, Ali A, et al. Pathogenicity of two Egyptian H5N1 highly pathogenic avian influenza viruses in domestic ducks. *Arch Virol* 2011;156:37–51.
- [19] Hulse-Post DJ, Franks J, Boyd K, Salomon R, Hoffmann E, Yen HL, et al. Molecular changes in the polymerase genes (PA and PB1) associated with high pathogenicity of H5N1 influenza virus in mallard ducks. *J Virol* 2007;81:8515–24.
- [20] Schat KA, Bingham J, Butler JM, Chen LM, Lowther S, Crowley TM, et al. Role of position 627 of PB2 and the multibasic cleavage site of the hemagglutinin in the virulence of H5N1 avian influenza virus in chickens and ducks. *PLoS One* 2012;7:e30960.
- [21] Song J, Feng H, Xu J, Zhao D, Shi J, Li Y, et al. The PA protein directly contributes to the virulence of H5N1 avian influenza viruses in domestic ducks. *J Virol* 2011;85:2180–8.
- [22] Qian C, Chen S, Ding P, Chai M, Xu C, Gan J, et al. The immune response of a recombinant fowlpox virus coexpressing the HA gene of the H5N1 highly pathogenic avian influenza virus and chicken interleukin 6 gene in ducks. *Vaccine* 2012;30:6279–86.
- [23] Hulse-Post DJ, Sturm-Ramirez KM, Humberd J, Seiler P, Govorkova EA, Krauss S, et al. Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. *Proc Natl Acad Sci USA* 2005;102:10682–7.
- [24] Steensels M, Van Borm S, Lambrecht B, De Vriesse J, Le Gros FX, Bublot M, et al. Efficacy of an inactivated and a fowlpox-vectored vaccine in Muscovy ducks against an Asian H5N1 highly pathogenic avian influenza viral challenge. *Avian Dis* 2007;51:325–31.
- [25] Cattoli G, Monne I, Fusaro A, Joannis TM, Lombin LH, Aly MM, et al. Highly pathogenic avian influenza virus subtype H5N1 in Africa: a comprehensive phylogenetic analysis and molecular characterization of isolates. *PLoS One* 2009;4:e4842.
- [26] Kim JK, Negovetich NJ, Forrest HL, Webster RG. Ducks: the Trojan horses of H5N1 influenza. *Influenza Other Respi Viruses* 2009;3:121–8.
- [27] Keawcharoen J, van Riel D, van Amerongen G, Bestebroer T, Beyer WE, van Lavieren R, et al. Wild ducks as long-distance vectors of highly pathogenic avian influenza virus (H5N1). *Emerg Infect Dis* 2008;14:600–7.
- [28] Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Humberd J, Seiler P, Putthavathana P, et al. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia. *J Virol* 2005;79:11269–79.
- [29] Gilbert M, Chaitaweesub P, Parakamawongsa T, Premashthira S, Tiensin T, Kalpravidh W, et al. Free-grazing ducks and highly pathogenic avian influenza, Thailand. *Emerg Infect Dis* 2006;12:227–34.
- [30] Songserm T, Jam-on R, Sae-Heng N, Meemak N, Hulse-Post DJ, Sturm-Ramirez KM, et al. Domestic ducks and H5N1 Influenza Epidemic, Thailand. *Emerg Infect Dis* 2006;12:575–81.
- [31] Henning J, Wibawa H, Morton J, Usman TB, Junaidi A, Meers J. Scavenging ducks and transmission of highly pathogenic avian influenza, Java, Indonesia. *Emerg Infect Dis* 2010;16:1244–50.
- [32] Chen H, Deng G, Li Z, Tian G, Li Y, Jiao P, et al. The evolution of H5N1 influenza viruses in ducks in southern China. *Proc Natl Acad Sci USA* 2004;101:10452–7.
- [33] Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, et al. Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. *Nature* 2004;430:209–13.
- [34] Swayne DE. Principles for vaccine protection in chickens and domestic waterfowl against avian influenza: emphasis on Asian H5N1 high pathogenicity avian influenza. *Ann NY Acad Sci* 2006;1081:174–81.
- [35] Beato MS, Toffan A, De Nardi R, Cristalli A, Terregino C, Cattoli G, et al. A conventional, inactivated oil emulsion vaccine suppresses shedding and prevents viral meat colonization in commercial (Pekin) ducks challenged with HPAI H5N1. *Vaccine* 2007;25:4064–72.
- [36] Long NT, Thanh TT, van Doorn HR, Vu PP, Dung PT, Dung TT, et al. Recent avian influenza virus A/H5N1 evolution in vaccinated and unvaccinated poultry from farms in Southern Vietnam, January–March 2010. *Transbound Emerg Dis* 2011;58:537–43.
- [37] Swayne DE, Pavade G, Hamilton K, Vallat B, Miyagishima K. Assessment of national strategies for control of high-pathogenicity avian influenza and low-pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. *Rev Sci Tech* 2011;30:839–70.
- [38] Desvaux S, Grosbois V, Pham TT, Dao DT, Nguyen TD, Fenwick S, et al. Evaluation of the vaccination efficacy against H5N1 in domestic poultry in the Red River Delta in Vietnam. *Epidemiol Infect* 2012;1–13.
- [39] Henning J, Henning KA, Morton JM, Long NT, Ha NT, Vu le T. Highly pathogenic avian influenza (H5N1) in ducks and in-contact chickens in backyard and smallholder commercial duck farms in Viet Nam. *Prev Vet Med* 2011;101:229–40.
- [40] Chen H. Avian influenza vaccination: the experience in China. *Rev Sci Tech* 2009;28:267–74.
- [41] Cagle C, To TL, Nguyen T, Wasilenko J, Adams SC, Cardona CJ, et al. Pekin and Muscovy ducks respond differently to vaccination with a H5N1 highly pathogenic avian influenza (HPAI) commercial inactivated vaccine. *Vaccine* 2011;29:6549–57.
- [42] Cagle CJW, Adams SC, Cardona CJ, To TL, Nguyen T, Spackman E, et al. Differences in pathogenicity, response to vaccination, and innate immune responses in different types of ducks infected with a virulent H5N1 highly pathogenic avian influenza virus from Vietnam. *Avian Dis* 2012;56:479–87.
- [43] Swaine DE, Senne DA, Beard CW. Avian influenza. In: Swaine DE, Jackwood MW, Pearson JE, Reed WM, editors. *A laboratory manual for the isolation and identification of avian pathogens*. 4th ed. Kennett Square, PA: American Association of Avian Pathologists; 1998. p. 150–5.
- [44] Das A, Spackman E, Pantin-Jackwood MJ, Suarez DL. Removal of real-time reverse transcription polymerase chain reaction (RT-PCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of Avian influenza virus by RT-PCR. *J Vet Diagn Invest* 2009;21:771–8.
- [45] Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J Clin Microbiol* 2002;40:3256–60.
- [46] Wan XF, Nguyen T, Davis CT, Smith CB, Zhao ZM, Carrel M, et al. Evolution of highly pathogenic H5N1 avian influenza viruses in Vietnam between 2001 and 2007. *PLoS One* 2008;3:e3462.
- [47] Swaine D.Ea.M.P.-J. Pathogenicity of avian influenza viruses in poultry. *Dev Biol (Basel)* 2006;124:61–7.

- [48] Pfeiffer J, Suarez DL, Sarmento L, To TL, Nguyen T, Pantin-Jackwood MJ. Efficacy of commercial vaccines in protecting chickens and ducks against H5N1 highly pathogenic avian influenza viruses from Vietnam. *Avian Dis* 2010;54:262–71.
- [49] Tian G, Zhang S, Li Y, Bu Z, Liu P, Zhou J, et al. Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics. *Virology* 2005;341:153–62.
- [50] Middleton D, Bingham J, Selleck P, Lowther S, Gleeson L, Lehrbach P, et al. Efficacy of inactivated vaccines against H5N1 avian influenza infection in ducks. *Virology* 2007;359:66–71.
- [51] Webster RG, Webby RJ, Hoffmann E, Rodenberg J, Kumar M, Chu HJ, et al. The immunogenicity and efficacy against H5N1 challenge of reverse genetics-derived H5N3 influenza vaccine in ducks and chickens. *Virology* 2006;351:303–11.
- [52] van der Goot JA, van Boven M, Stegeman A, van de Water SG, de Jong MC, Koch G. Transmission of highly pathogenic avian influenza H5N1 virus in Pekin ducks is significantly reduced by a genetically distant H5N2 vaccine. *Virology* 2008;382:91–7.
- [53] Swayne DE. The role of vaccines and vaccination in high pathogenicity avian influenza control and eradication. *Exp Rev Vaccinol* 2012;11:877–80.
- [54] Kim JK, Seiler P, Forrest HL, Khalenkov AM, Franks J, Kumar M, et al. Pathogenicity and vaccine efficacy of different clades of Asian H5N1 avian influenza A viruses in domestic ducks. *J Virol* 2008;82:11374–82.
- [55] OFFLU. An OFFLU agenda for influenza research priorities in animal species; 2011.
- [56] Swayne DE, Garcia M, Beck JR, Kinney N, Suarez DL. Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. *Vaccine* 2000;18:1088–95.
- [57] Swayne DE, Perdue ML, Beck JR, Garcia M, Suarez DL. Vaccines protect chickens against H5 highly pathogenic avian influenza in the face of genetic changes in field viruses over multiple years. *Vet Microbiol* 2000;74:165–72.
- [58] Lee CW, Senne DA, Suarez DL. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. *J Virol* 2004;78:8372–81.
- [59] Smith DJ, Lapedes AS, de Jong JC, Bestebroer TM, Rimmelzwaan GF, Osterhaus AD, et al. Mapping the antigenic and genetic evolution of influenza virus. *Science* 2004;305:371–6.